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Multistage Countercurrent Distribution

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Theory

The separation of chemical compounds by partitioning between two liquid phases, so-called liquid–liquid extraction, can be made more effective by using it as a cascade process. One way in which this can be carried out is by multiplicative partitioning, also called countercurrent distribution (CCD). This process, in which complete partition equilibrium is achieved in each step, is presented schematically in **Figure 1**. The principle is that two sets of liquid phases, the upper and lower phase, come into contact with each other stepwise. The bottom phases are numbered 0, 1, 2 and so on. The sample to be analysed (fractionated) is included in the first system (containing bottom phase number 0). Before each transfer of the upper phases (to the right in Figure 1) the two-phase systems are equilibrated by mixing and the sample components are distributed between each pair of phases (each full two-phase system). The partitioning of a pure substance between the phases of a two-phase system can be expressed either by a partition coefficient, K, defined as the ratio of the concentrations (C) of the component in the phases:

$$K = \frac{C\ (\text{in phase I})}{C\ (\text{in phase II})} \qquad [1]$$

or by a partition ratio, G, defined as the ratio of the masses (m) of the components in the phase:

$$G = \frac{m\ (\text{in phase I})}{m\ (\text{in phase II})} \qquad [2]$$

K and G are related by eqn [3]:

$$G = K\frac{\text{Volume (phase I)}}{\text{Volume (phase II)}} \qquad [3]$$

In the following the upper phase is chosen as phase I. A convenient way of analysing the CCD process is to calculate the amounts (in fractions) of a pure

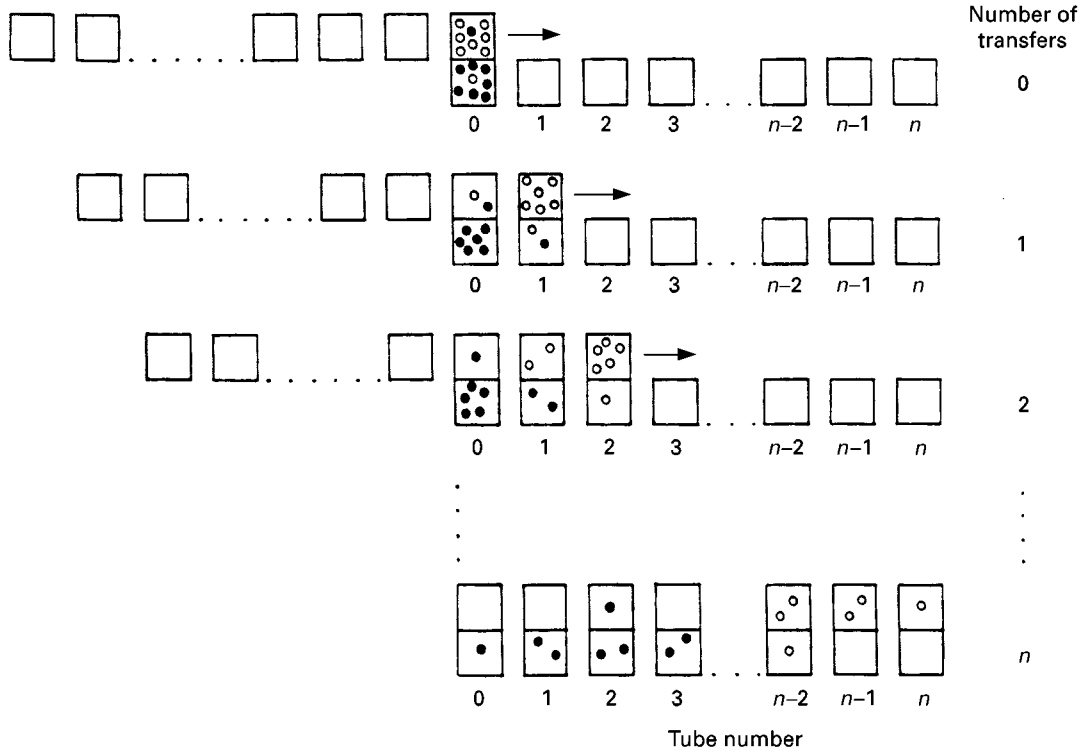

Figure 1 Principle of CCD. The upper phases are transferred stepwise from left to right. Between each transfer the two-phase systems are equilibrated by shaking and the phases are allowed to settle. Filled circle, substance with small *G* value; open circle, substance with large *G* value.

component in the various phases based on its *G* value. In the initial two-phase system (number 0) the component is distributed with the fractions p in the upper phase (phase I) and q in the lower phase (phase II). By definition $p + q = 1$. When the upper phase of system number 0 is combined with the pure lower phase number 1 equilibration of this system will make the transferred fraction p distribute as p/q ($= G$) giving the fraction p^2 in the upper phase and the fraction pq in the lower phase. In the new system number 0, with a new upper phase, the remaining fraction q will partition likewise and the equilibration results in the fraction pq in the upper phase and q^2 in the lower phase. The resulting distribution after 10 transfers in the 11 tubes 0–10 is shown in **Figure 2**. In the same figure the material in each tube (upper plus lower phase) has been calculated. These values are the same as the terms obtained when $(q + p)^{10}$ is written as a polynomial. More generally, the amount of material $T_{n,i}$ (in fractions), in tube number i after a CCD with n transfers is given by eqn [4]:

$$T_{n,i} = \frac{n!}{i!(n-i)!} p^i q^{n-i} \qquad [4]$$

The volume ratio is kept constant during the process. By using the relations $G^i = p^i/q^i$ and $(1 + G)^n =$

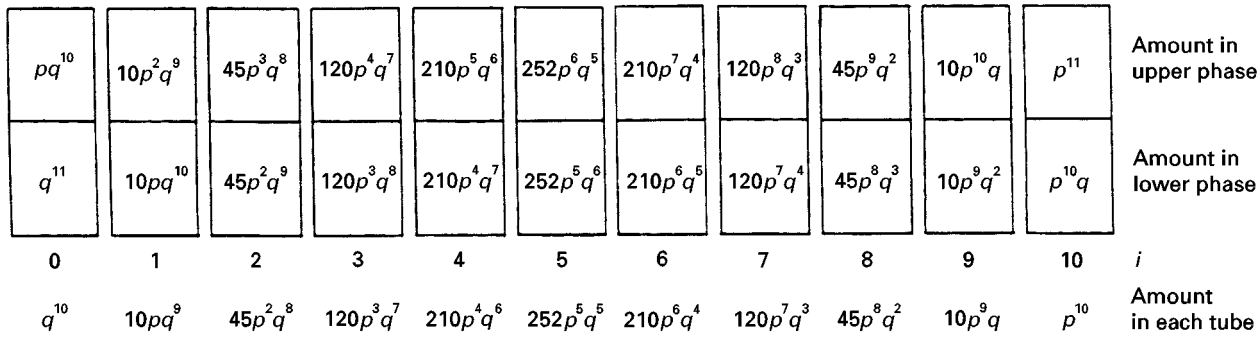

Figure 2 Distribution of a component which partitions in the ratio p/q between the upper and lower phase – after a CCD with 10 transfers. The amounts are given in fractions, i.e. $p + q = 1$.

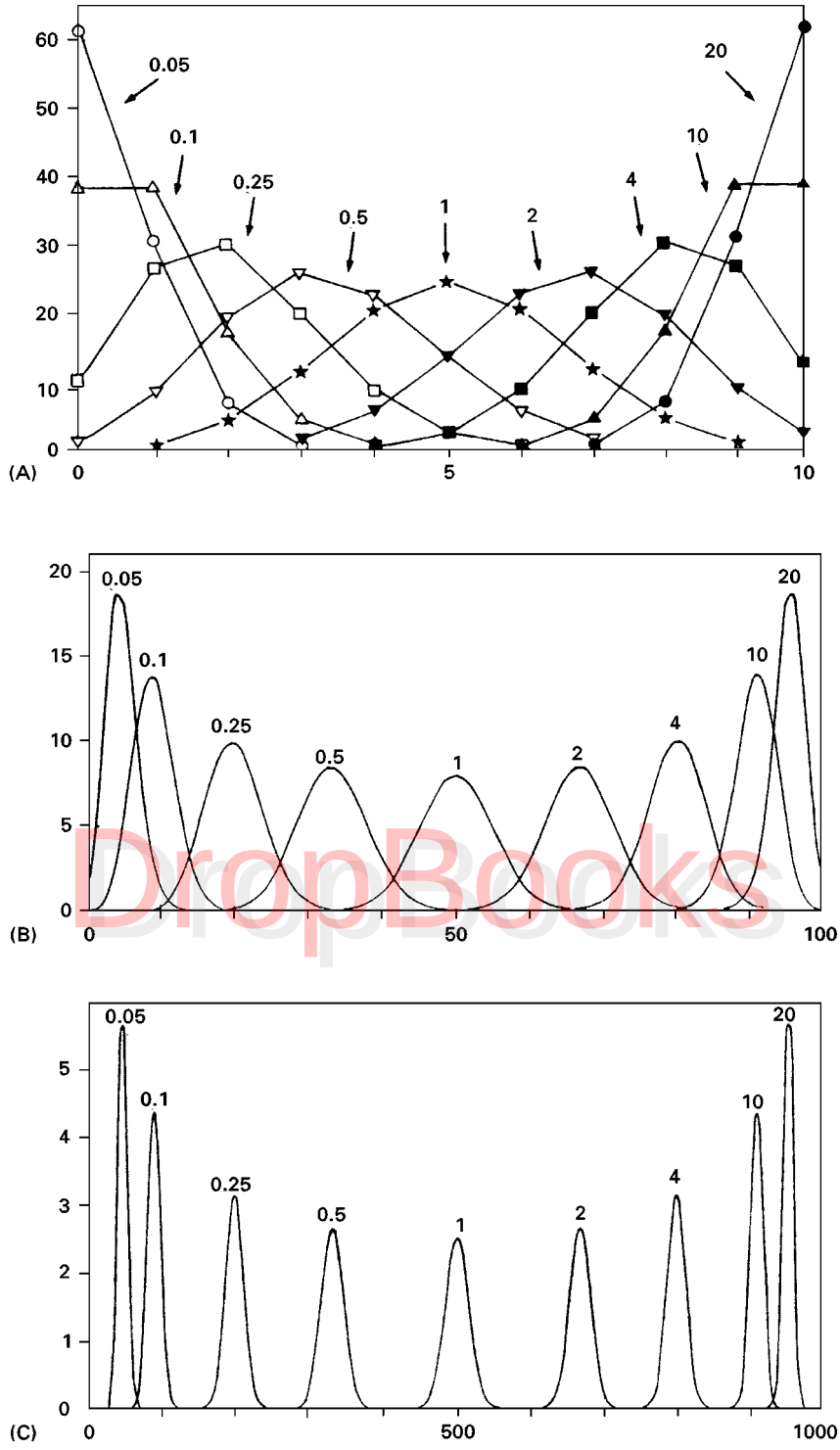

Figure 3 The resulting CCD profiles after (A) 10 transfers, (B) 100 transfers and (C) 1000 transfers for substances with the indicated G values.

$(1/q)^n$, eqn [5] is obtained:

$$T_{n,i} = \frac{n!}{i!(n-i)!}\frac{G^i}{(1+G)^n} \qquad [5]$$

This relationship has been used to calculate the distribution profiles in **Figure 3** for three cases: i.e. with the number of transfers (n) equal to 10, 100 and 1000. In each case the distributions of components with

G values of 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 10 and 20 are shown. The resolution of components with various G values increases with the increase in the number of transfers. This is due to the fact that the difference in the position (tube number) of the peaks (with given G values) is proportional to the number of transfers, while the peak width only increases with the square root of the number of transfers.

The width of a peak Δr, covering 99.7% of the compound, can be approximately calculated using eqn [6]:

$$\Delta r = \frac{6\sqrt{nG}}{(1+G)} \qquad [6]$$

The relative width of a peak, $\Delta r_{rel} = \Delta r/n$, is given in **Table 1** for $G = 1$ and a number of n values.

The most effective separation of two components, with partition coefficients K_1 and K_2, is achieved when their distribution peaks are oriented symmetrically around the middle of the tube train, i.e. tube $i = n/2$. This, as can be seen in **Figure 3**, is equal to the relation $G_1 = 1/G_2$ which can be written as $G_1 \times G_2 = 1$. By combining this relation with eqn [3] the volume ratio, V, to be used for optimal separation can be calculated as shown in eqn [7]:

$$V = \frac{1}{\sqrt{K_1 K_2}} \qquad [7]$$

The ratio between the K values of two substances 1 and 2 is called the separation factor, β, and is a measure of the separability of the substances. In **Table 2** the number of transfers necessary for virtually complete (more than 99.5%) separation has been calculated for various β values.

The G value of a component which has its maximum peak value in tube number $\hat{\imath}$ after n transfers is given by the approximate eqn [8]:

$$G = \hat{\imath}/(n - \hat{\imath}) \qquad [8]$$

Table 1 The absolute, Δr, and relative, Δr_{rel}, peak width (percentage of the total number of tubes) of a substance with $G = 1$ after a CCD with n transfers

n	Δr *(no. of tubes)*	Δr_{rel} (%)
20	13	67
50	21	42
100	30	30
200	42	21
500	67	13
1000	95	9.5
2000	134	6.7
5000	212	4.2

Table 2 Number of transfers, n, in a CCD necessary to separate two substances, 1 and 2, with a given separation factor $\beta = K_1/K_2$. The volume ratio is chosen in such a way (eqn [7]) that $G_1 \times G_2 = 1$

β	G_1	G_2	n
2	0.709	1.414	264
3	0.577	1.732	110
4	0.500	2.00	70
9	0.333	3.00	30
16	0.250	4.00	20
25	0.200	5.00	16
36	0.167	6.00	14
49	0.143	7.00	12
81	0.111	9.00	10
400	0.050	20.0	6
2500	0.020	50.0	4

The G value can also be obtained by comparing the amount of a substance in two consecutive tubes, numbers i and $i + 1$. By combining the expressions for $T_{n,i}$ and $T_{n,i+1}$ obtained from eqn [5] the G value is obtained as in eqn [9]:

$$G = \frac{T_{n,i+1}}{T_{n,i}} \frac{i+1}{n-i} \qquad [9]$$

By following the apparent G values over a distribution peak the presence of several substances differing slightly in their G values can be detected.

Various Modes of Multiplicative Partitioning

The basic process of CCD has been described, but it can be varied in a number of ways.

Single Withdrawal Procedure

In this process the addition of fresh upper phases is not stopped after n transfers, but continues from the left while the overflowing upper phases (to the right) are collected. This process is therefore similar to a chromatographic system with the lower phases corresponding to the stationary phase of the column and the upper phases corresponding to the elution liquid. Substances with high G values are easily eluted in this way while those with low G values need a great number of steps to be carried through the CCD train. The greater the number of transfers the more diluted the substance will be when leaving with the overflowing upper phases. Likewise the upper phases can be chosen as the stationary phase and the lower phases can be used as the eluting phases. In this case substances with low G values rapidly eluted.

O'Keefe Partitioning

The multiplicative partitioning can be carried in such a way that a new portion of the solute mixture to be separated is added after each transfer step. This method of carrying out the partitioning is called O'Keefe partitioning. Several two-phase systems are arranged in a row and a solute sample is included in the centre system. After equilibration and settling all the upper phases are moved one step to the right and the overflowing upper phase is collected. Then all the lower phases are moved one step to the left and the one to the extreme left is collected. The remaining set is completed with one fresh lower phase (to the right) ane one fresh upper phase (to the left) to restore the original number of systems. After addition of a new portion of solute to the centre system the next cycle takes place. Substances with $G > 1$ will be recovered in the upper phases collected to the right and substances with $G < 1$ in the lower phases removed to the left. This process is used when large amounts of substances are to be separated. The number of systems is usually small, e.g. 7, 9 or 11.

A similar process, called Watanabe–Morikawa partitioning, differs in that the ongoing addition of solute mixture is done in the first system of the row. This is useful when only components with high or low G values are to be isolated.

Description of Some CCD Apparatus

CCD with only a few transfers (up to 10) can easily be carried out by using a set of separating funnels for mixing, settling and phase separation. For small phase volumes, 0.5–5 mL, the separating funnels can be replaced by test tubes and the upper phases can be transferred with the aid of a pipette. For CCDs with more than 10 transfers it is strongly advised that some kind of automatic apparatus is used. Some examples of such apparatuses are presented next.

All-Glass CCD According to Craig

Several glass units, allowing mixing, settling and phase transfers (**Figure 4**) are arranged in batteries on a horizontal axis. Movement about this axis can be used to gently mix the phases, to put the glass units in position for settling of the phases and for decanting the upper phases, respectively. The times for each part of the CCD cycle as well as the number of transfers can be programmed. Standard types of such machines allow 50 transfers while more advanced machines may be used for up to 1000 transfers. The glass units can be obtained in various sizes but standard tubes have space for *c.* 2 mL (fixed amount) of lower phase and up to 5 mL of upper phase.

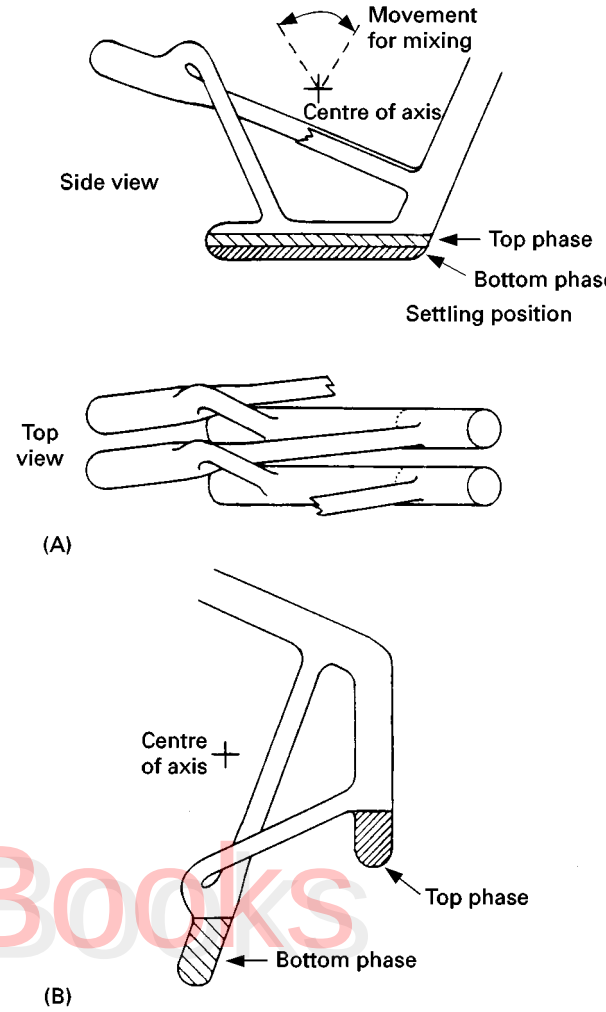

Figure 4 Section of the all-glass apparatus for CCD constructed by Craig. (A) Two segments (or tubes) are shown from side and top views. Normally 50–1000 tubes are connected and placed on a horizontal rack which allows simultaneous mixing of all tubes. (B) By turning the axis the upper phases can be decanted. When returning to the original position the upper phases are transferred to the neighbouring tube.

Thin-Layer CCD According to Albertsson

This CCD apparatus is designed to be used with aqueous two-phase systems which normally need a long time for settling. By using systems with low heights, only a few millimetres, the settling time will be acceptable (5–20 min). The operating unit (**Figure 5**) consists of two circular plates of polyacrylic plastic, one on top of each other: the lower plate (stator) is fixed while the upper one (rotor) can be rotated stepwise. Both plates have 60 (or 120) radially oriented cavities which pair-wise form containers for the two-phase systems. The cavities of the stator contain the lower phases (normally 0.8 mL) while the upper phases (0.2–2 mL) are situated in the

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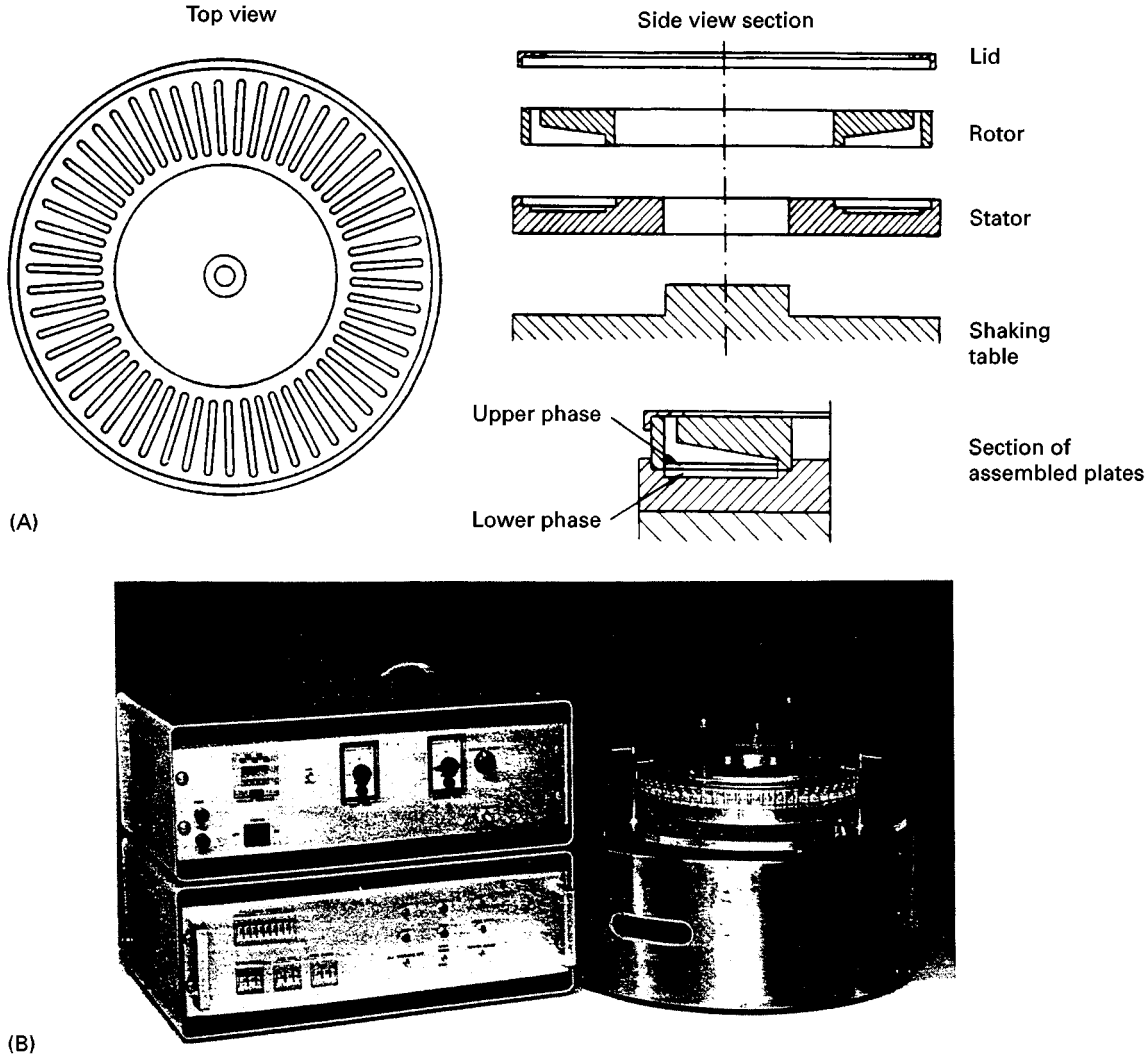

Figure 5 Thin-layer CCD apparatus according to Albertsson. (A) The two discs of polyacrylic plastic contain matching cavities for the upper and lower phases and by rotation the upper plate can move all upper phases relative to the lower ones. See text for further details. (Adapted from Albertsson P-Å (1986) *Partition of Cell Particles and Macromolecules,* 3rd edn, p. 126. New York: Wiley (interscience), by permission.) (B) A thin-layer CCD machine constructed by Albertsson.

cavities of the rotor and therefore can be transferred relative to the lower phases by rotating the upper plate. The two plates are placed on the shaking table of a machine which also contains a drive for the upper plate rotation. The phase systems are added to each container (chamber) via openings in the upper side of the rotor. These openings, during the run, are covered with a ring-formed lid. The shaking and settling periods and number of transfers are controlled by an automatic unit. After the CCD run is completed the two-phase systems can be collected by use of a fraction collector. This is a circular rack with the same number of test tubes (4 mL) as chambers. The rack is placed over the inlet holes and is inverted together with the plates which are then emptied.

Centrifugal CCD According to Åkerlund

This kind of CCD machine uses centrifugation to speed up the settling of the phases. It consists of an outer ring with cavities for the lower phases (attached to a bottom plate) and an inner plate with cavities for the upper phases (**Figure 6**.) The inner plate can rotate relative to the outer ring. When the chambers have been loaded with systems they are covered with a lid. As in the case of the thin-layer machine the functional unit is placed on a (round) table which can be shaken. In this case, however, the table can also rotate to allow centrifugation of the mixed systems which speeds up the settling of the phases. The upper phases are then transferred to the neighbouring lower

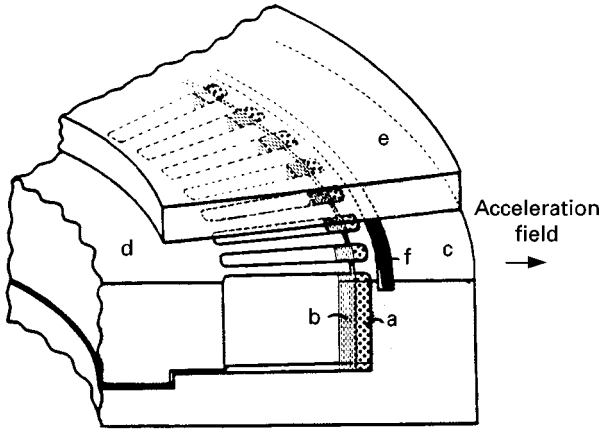

Figure 6 Centrifugal CCD according to Åkerlund. A section of the circular separation unit is shown. It is composed of four units: c, the outer ring with cavities for the lower phase: d, the inner plate with cavities for the upper phase; e, the lid; and f, an O-ring for sealing. The position of the two-phase system during centrifugation is shown, with the upper phase, a, and the lower phase, b. (From Johansson G, Åkerlund H-E and Olde B (1984) *Journal of Chromatography* 311: 277, by permission.)

phases by rotation of the inner plate when the whole system is still spinning and the phases are in a vertical position. After the transfer the centrifugation is stopped and a new cycle begins with the shaking process. After the run the phases are collected by pipette.

Continuous apparatus, such as extraction columns and coil planet centrifuges, also corresponds to a CCD process but differs in that no discrete steps are used and equilibrium is not reached.

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Analytical Applications

Many metal ions can be separated by using various kinds of water/organic solvent systems for countercurrent distribution. Metal chelators, such as dithizone, 8-quinolinol, cupferron, dimethylglyoxime and acetylacetone, are used specifically to extract metal ions into the organic phase. Likewise certain anions such as halides, thiocyanate or nitrate can be used. For example, uranium and plutonium have been separated by using an aqueous phase containing 8 mol L^{-1} nitric acid.

Biochemical Applications

CCD has been used for fractionation of a number of biochemical substances and cellular particles as well as cells and viruses. Peptides, proteins and nucleic acids have been fractionated by using aqueous two-phase systems composed of water and the two polymers dextran and poly(ethylene glycol) (PEG). The partition coefficients of the solutes can be adjusted by addition of various salts to the two-phase system and by adjusting the pH value. More selective adjustment of the partitioning has been carried out by linking an affinity ligand to one of the phase-forming polymers which then is concentrated in one of the phases.

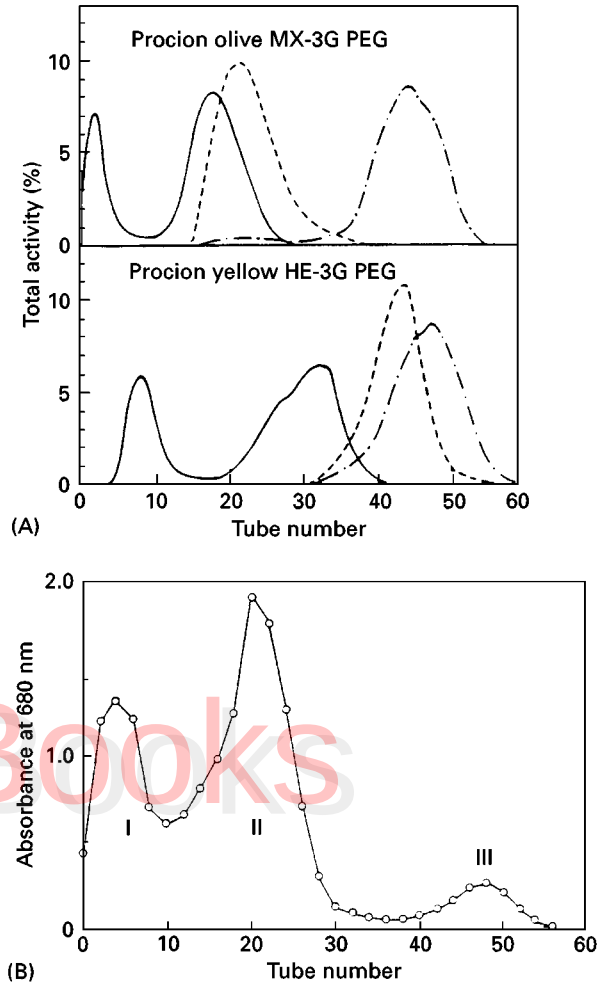

Figure 7 Countercurrent distribution of proteins and cell organelles. (A) Distribution of the enzymes hexokinase (—), 3-phosphoglyceratekinase (- - -), and phosphofructokinase (-·-·-·-) when an extract of baker's yeast was applied to a CCD with 55 transfers. The textile dyes Procion olive MX-3G or Procion yellow HE-3G were used as PEG-bound affinity ligands enriched in the mobile upper phase. Composition of two-phase system: 88% (w/w) water, 7% (w/w) dextran 500, 5% PEG with $M_r = 8000$, including dye-PEG, 1% of total PEG 50 mmol L^{-1} sodium phosphate buffer, pH 7.0, 0.2 mmol L^{-1} ethylenediaminetetraacetic acid and 5 mmol L^{-1} 2-mercaptoethanol. Volume ratio, 1.5. Temperature, 3°C. A centrifugal CCD apparatus was used with 5 min shaking and 3 min settling (centrifugation). (Adapted from Johansson G, Joelsson M and Åkerlund H-E (1984) *Journal of Chromatography* 298: 483, by permission.) (B) Fractionation of photosynthetic particles, chloroplasts, from spinach using a thin-layer CCD apparatus with 56 transfers and a dextran–PEG two-phase system. Peak I, intact chloroplasts surrounded by their envelope; peak II, naked thylakoid membranes (class II chloroplasts); peak III, choloroplasts surrounded by a 'bag' of plasma membrane also containing cytoplasm, mitochondria and peroxisomes. (From Larsson C, Collin C and Albertsson P-Å (1971) *Biochimica Biophysica Acta* 245: 425, by permission.)

Biological membranes, cell organelles, whole cells and viruses can be fractionated by CCD in the same kind of systems. In this case, however, the particles partition between the two liquid phases and the interface between them. The CCD is therefore usually carried out using a stationary interface. This is achieved by using a smaller volume of the lower phase than is needed to fill the lower cavities. Therefore, a portion of the upper phases will also be stationary. The G value satisfying eqn [5] is in this case defined as the amount of a pure compound, at equilibrium, in the mobile part of the upper phase divided by the amount of the compound in the rest of the system (stationary upper phase, interface and lower phase). Examples of CCD of proteins and of chloroplasts, the photosynthetic organelle in green plant cells, are given in **Figure 7**.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation.

Further Reading

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Solid-Phase Extraction

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Solid-phase extraction is a method used to isolate analytes from a gas, fluid or liquid by their transfer to and retention on a solid-phase sorbent. After separation of the sorbent from the sample the analytes are recovered by elution using a liquid or fluid, or by thermal desorption into the gas phase. If the analytes are recovered from the sorbent in a final volume that is only a fraction of the sample volume, then concentration as well as isolation is achieved. In addition, if the sorption step, any subsequent rinse steps, and the elution conditions are selective for retention and recovery of the analyte, then matrix simplification is achieved. Isolation, concentration and matrix simplification are the primary goals of solid-phase extraction.

Probably the earliest application of solid-phase extraction was the use of charcoal-filled columns in the 1950s to isolate organic contaminants from surface waters for toxicity evaluation. The large volume of water generally sampled (more than 1000 L over several days) precluded the use of liquid–liquid extraction techniques. The subsequent evolution of solid-phase extraction techniques is summarized in **Figure 1**.

The introduction of macroreticular porous polymers in the early 1970s was responsible for rekindling interest in solid-phase extraction and extending its scope to air sampling and the isolation of drugs from biological fluids. These sorbents had reasonable mechanical strength compared with gels, a large surface area and sample capacity, low water retention, and gave high sample recoveries by solvent desorption. Compared with carbon the overall analyte recovery was generally better and irreversible adsorption and catalytic activity greatly diminished. These

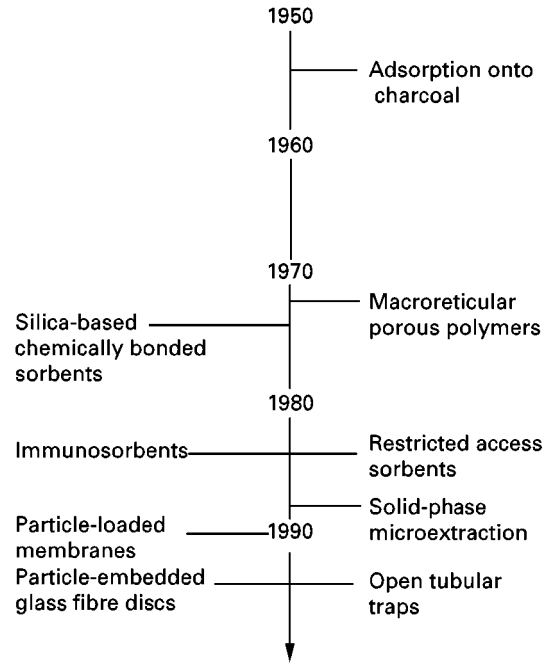

Figure 1 Time line showing the general evolution of solid-phase extraction techniques.